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Molecular Genetic Characteristics of Nepalese *Ephedra* Plants (*Ephedraceae*)

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The aerial part of *Ephedra* plants is a staple crude drug worldwide. With the aim of developing new *Ephedra* resources, we investigated Nepalese wild *Ephedra* plants, *E. gerardiana* Wall. ex Stapf and *E. pachyclada* Boiss., and analyzed their molecular genetic features by DNA analysis. *Ephedra gerardiana* were distributed on the sub-Himalayan steppes at high altitudes, 3100–4500 m, and *E. pachyclada* were found along the Kali Gandaki River Valley in the Mustang region at relatively lower altitudes, 2000–3500 m. *Ephedra gerardiana* specimens from western collection sites shared an identical ITS1 sequence, while that of specimens from eastern collection sites at higher altitudes was 1–12 bases different from western specimens. *Ephedra pachyclada* specimens showed complex ITS1 signals by the overlapping of two type sequences. By cloning of ITS1 sequences of four *E. pachyclada* specimens from around the 400th nucleotide to separate these overlapping sequences, they were confirmed to contain sequences either monophyletic with *E. intermedia* Schrenk & C. A. Mey. or *E. gerardiana*. The ratio of clones with these two types varied among the specimens. Both Nepalese *Ephedra* plants, especially *E. pachyclada*, had a markedly heterogeneous ITS1 sequence that may be caused by harsh environmental pressure at high altitudes.

Key words: DNA, *Ephedra gerardiana*, *Ephedra pachyclada*, Himalaya, ITS1, Nepal.

The aerial part of *Ephedra* plants (*Ephedraceae*) is a staple crude drug worldwide. In Chinese and Japanese traditional medicines, *Ephedra* is used in prescriptions for its analgesic, antipyretic and antitussive effects, and its medicinal ingredients, ephedrine alkaloids, are contained in modern cold treatments. Likewise, crude drugs prepared from *Ephedra* plants, such as "TSHE" and "BALU", are used in Tibetan medicine, which is commonly practiced in the Himalayan region (Mikage et al. 1996, 1998). Two *Ephedra* plants, *E. gerardiana* Wall. ex Stapf and *E. pachyclada* Boiss., are reported to grow in Nepal (Mikage et al. 1996, 1998). *Ephedra gerardiana* is found widely in the sub-Himalayan steppe in high altitudes, 3100–4500 m, while *E. pachyclada* inhabits a limited area centering on the Mustang District at relatively lower altitudes, 2000–3500 m (Kondo et al. 1999). The worldwide distribution of the former

Table 1. Collection dates and localities of *Ephedra* specimens

	ID	Voucher(KANP)	Collection date	Sex	Site	Longitude (E)	Latitude (N)	Altitude(m)
E. gerardiana	EG1	9106101	1991/10/1	♀	S1	82°40′	29°10′	3340
	EG2	9106134	1991/10/6	♀	S2	82°50′	29°10′	3690
	EG3	932304	1993/7/31	♀	S3	83°39′	28°44′	3140
	EG4	932408	1993/7/31	♂	S3	83°39′	28°44′	3140
	EG5	9465030	1994/8/20	♀	S4	83°53′	28°49′	3800
	EG6	9465013	1994/8/18	♀	S5-1	83°58′	28°45′	4060
	EG7	8580458	1985/7/25	♂	S7	86°28′	27°45′	4150
	EG8	8581179	1985/8/26	♀	S8	86°36′	27°41′	3970 – 4450
	EG9	8860326	1988/7/22	♂	S9	87°05′	27°45′	4510
	EG10	9261147	1992/6/1	♂	S10	87°51′	27°46′	3260 – 4030
	EG11	9465018	1994/8/18	♂	S5-2	83°58′	28°45′	4215
	EG12	9460267	1994/8/8	♀	S6	84°46′	28°38′	4055
E. pachyclada	EP1	9105157	1991/10/15	uk	S11	82°50′	28°50′	2080
	EP2	932394	1993/8/5	♀	S12	83°37′	28°40′	2520
	EP3	932302	1993/7/31	♀	S13	83°38′	28°41′	2570
	EP4	932322	1993/8/1	♀	S14	83°41′	28°45′	2640
	EP5	933203	1993/8/1	♀	S15	83°42′	28°46′	2650
	EP6	932349	1993/8/2	♀	S16	83°48′	28°49′	2790
	EP7	932373	1993/8/4	♀	S17	83°50′	28°49′	3220
	EP8	932366	1993/8/3	♂	S18	83°52′	28°49′	3460
	EP9	20103202	2001/8/3	uk	S16	83°47′	28°50′	2740
	EP10	20103204	2001/8/3	uk	S16	83°47′	28°50′	2850
	EP11	20103227	2001/8/7	uk	S19	83°56′	29°05′	3280
	EP12	20315117	2003/6/3	uk	S20	84°00′	29°15′	nk

uk: unknown.

ranges from southwestern Asia, including Afghanistan, northern part of Pakistan and India, to southern China (USDA 2010, Cheng 1978). The latter overlaps with the former but in a narrower region in Iran and Afghanistan (http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl 15234 (03 March 2010), http://www.conifers.org/ep/ep/pachyclada.htm). Both contain ephedrine alkaloids; in particular, ephedrine and pseudoephedrine concentrations of the latter are comparable to the *Ephedra* plants prescribed in the Chinese and Japanese Pharmacopeias (Kondo et al. 1999). However, they should be specified precisely for effective and safe medicinal use. With the aim of developing new *Ephedra* resources, we investigated wild *Ephedra* plants in Nepal and analyzed their molecular genetic features by DNA analysis.

Materials and Methods

Plant materials

Ephedra plants were collected during field surveys in Nepal from 1985 through 2001 at the sites shown in Fig. 1. Observing the shape of micropyle tube of female cone (Stapf 1888), 12 specimens of each species, morphologically identified as *E. geradiana* and *E. pachyclada*, were used for DNA analysis (Table 1).

Abbreviations. ITS 1 and 2: The internal transcribed spacer 1 and 2 of nuclear ribosomal DNA, *trn L/F*: The *trnL* (UAA) intron and the intergenic spacer between the *trnL* (UAA) 3' exon and the *trnF* (GAA) gene of chloroplast DNA.

Total DNA isolation and PCR amplification

Isolation of total DNA: From dried herbarium specimens of the collected *Ephedra*

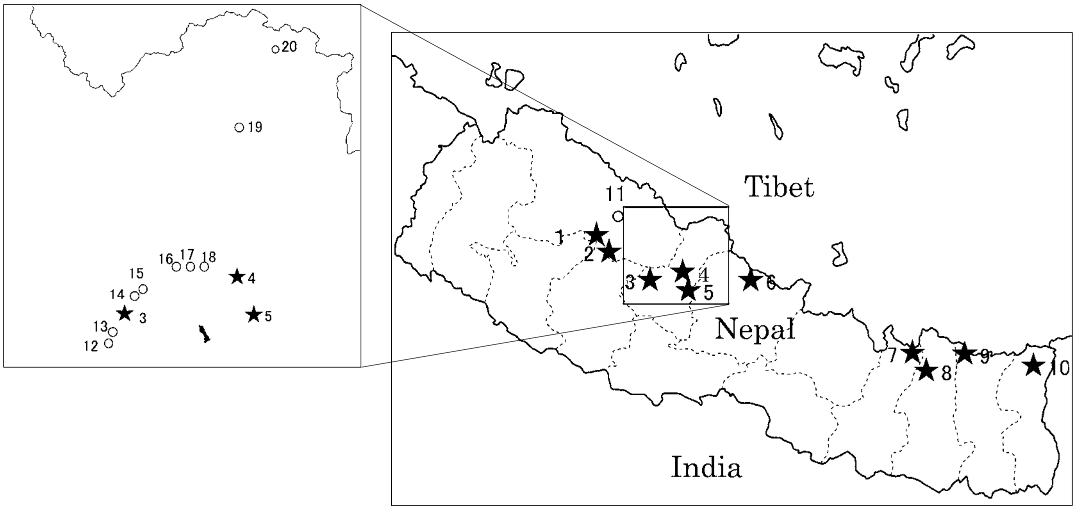


Fig. 1. Collection sites of *Ephedra* plants in Nepal. Star. *E. gerardiana*. Circle. *E. pachyclada*.

plants, about 20–50 mg stems were cut off, frozen in liquid nitrogen and ground into fine powder. Total DNA isolation from the powder was performed using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol.

Polymerase chain reaction (PCR) amplification: The ITS region was amplified by PCR using 30–100 ng total DNA as a template in 25 μl reaction mixture containing 2.5 μl of 10 × PCR buffer for KOD-Plus, 0.2 mM of each dNTP, 1.0 mM $MgSO_4$, 0.5 unit KOD-Plus-polymerase (Toyobo), and 0.4 μM primers. Total DNA was used as a template for amplifying ITS1, 2 and *trnL/F*. For amplification, the following primer sets were used; for ITS1: Eph-1F1 (GAC GTC GCG AGA AGT TCA TT)/ITS1B (CTA TGA TGT GCC AGG CAT CC) and ITS1A (GCG GGG ACG TGG ACG GTC TT)/5.8SR (CGG GAT TCT GCA ATT CAC AC), for ITS2: 5.8SF (GAA CGT AGC GAA ATG CGA TA) /Eph-1R (GTA AGT TTC TCT TCC TCC GC), and for *trn L/F* : Aco 1F (CGA AAR CGG TAG ACG CTA CG) / Aco2R (ATT TGA ACT GGT GAC ACG AG). Amplification was carried out under the following conditions: pre-heating at 94°C for 2 min, 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and elongation at 68°C for 45 s, with a final elongation at 68°C for 5 min. Three microliters of the PCR product were analyzed by agarose gel electrophoresis and then the remaining part was purified using a QIAquick PCR Purification Kit (Qiagen).

Sequencing procedure

The purified PCR product was subjected to direct sequencing using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) by ABI PRISM 310 (Applied Biosystems). The primers used for sequencing of ITS1, were ITS 1A, ITS N (GAC GTC GCG AGA AGT TCA TT), ITS O (GTC AAA GAC CGT CCA CG T CC), ITS 1D (CCC TTC CCC GTG TAA CAC GC), rev D (GCG TGT TAC A CG GGG AAG GG), 5.8SR. Their binding sites are indicating in Fig. 2. The other primers used were 5.8SF and Eph 1R for ITS2, and Aco 1F, Aco3F (CTA GAG AGA AAA GAA ATC CG), Aco1R (GGG ATA GAG GGA CTT GAA C) and Aco2R for *trn L/F*. The DNA sequences were aligned by 'DNASIS' version 3.0 (Hitachi).

Cloning of PCR products

Total DNA was amplified by PCR using primer sets BH ITS1A (AAA AGG ATC CGC

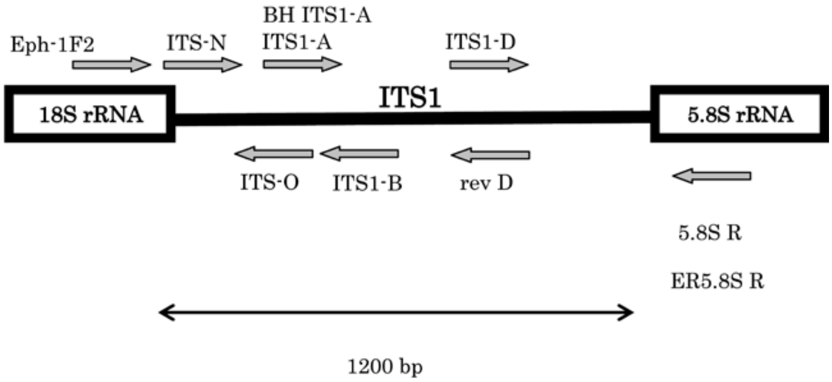


Fig. 2. Binding sites of primers in ITS1.

GGG GAC GTG GAC GGT CTT) / ER 5.8SR (TAA TGA ATT CCG GGA TTC TGC AAT TCA CAC) for ITS1 and BH Aco1F (TCT TGG ATC CGA ARC GGT AGA CGC TAC G) /HD Aco2R (CTC CAA AGC TTT GAA CTG GTG ACA CGA G) for *trn L/F*. After purification using a QIAquick PCR purification kit, 100–200 ng PCR products were digested with Bam HI and Eco RI or Hind III (Takara Biotech) in a 10 µl reaction mixture containing 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM NaCl at 37°C for 1 hr, and then the reaction mixture was heated at 70°C for 15 min. Four microliters of the reaction mixture was combined with 5 ng plasmid pBluescript SK (−) digested with the same enzymes and 5 µl DNA Ligation Kit Ver.2.1 (Takara Biotech). The ligation reaction was performed at 16°C overnight. Competent cells (Competent high DH5α; Toyobo) were transformed with the ligated product, as recommended by the manufacturer's protocol. The transformed cells were spread on Luria broth (LB) agar plates containing ampicillin (Amp) (2% tryptone, 1% yeast extract, 2% NaCl, 3% agar, and 100 µg/ml ampicillin) and incubated at 37°C overnight. Bacterial colonies were picked up separately, and sub-cultured in liquid LB / Amp medium. After overnight incubation, the bacteria were collected by centrifugation and plasmids were obtained following the standard protocol (Sambrook et al. 1989) with minor modification (Kitaoka et al. 2009). Sequencing of plasmids was performed following the method stated in the previous section.

Results

Nucleotide variation in ITS1 of Nepalese *Ephedra gerardiana*

The collection sites of a group of specimens morphologically identified as *E. gerardiana* were distributed on the sub-Himalayan steppes at high altitudes, 3100–4500 m (Table 1, Fig. 1). They had ITS1 sequences similar to those of Chinese (Long et al. 2004) and Pakistan (Kakiuchi et al. 2007) *E. gerardiana*. Specimens EG 1–5 from western collection sites S1–S4 shared an identical ITS1 sequence that differed from Chinese *E. gerardiana* and Pakistan *E. gerardiana* at 4 and 1 positions, respectively (Table 2). On the other hand, the specimens EG 7-9 from eastern collection sites at higher altitudes, S6-S9, differed by 1–14 bases from western specimens. Two specimens, EG6 and EG11, collected from close collection sites at different altitudes (S5-1 and from S5-2, respectively) had similar ITS1, but the latter showed overlapping signals of western and eastern sequences. Specimen EG10 from S10, a collecting site near the eastern border, had

Table 2. Alignment of ITS1 sequences of *Ephedra* plants morphologically identified as *E. gerardiana*

Specimen ID (site)	33	83	67–97	139	145	181	217	219	242	269	325	327	393	401–03	472	478	500	511	538	539	567	583	593	643	669
E. intermedia (China) [a)]	C	T	AA	T	G	T	T	G	A	C	G	A	A	AAA	T	T	C	T	A	G	A	A	C	C	T
E. gerardiana (China) [b)]	Y	A	TG	G	-	C	C	*	*	T	*	*	C	---	C	C	*	C	G	A	C	T	*	-	C
E. gerardiana (Pakistan) [c)]	*	A	TG	G	-	C	C	*	*	T	*	*	C	---	C	C	*	C	G	*	C	T	G	*	*
EG1, EG2, EG3, EG4, EG5, EG6, EG10	T	A	TG	G	-	C	C	*	*	T	*	*	C	---	C	C	*	C	G	*	C	T	*	*	*
EG7	T	A	TG	G	-	C	C	*	*	T	*	*	C	---	C	C	*	C	G	*	C	T	*	*	*
EG8	*	A	TG	G	-	C	C	A	G	T	T	A	C	---	*	*	*	*	*	*	*	*	*	*	*
EG9	*	A	TG	G	-	C	C	A	G	T	T	A	C	---	C	C	*	C	G	*	C	T	*	*	*
EG11	T	A	TG	G	-	C	C	*	*	T	*	*	C	---	C	C	A	C	G	*	C	T	*	*	*
EG12	*	A	TG	G	-	C	C	A	G	T	*	*	C	---	C	C	*	C	G	*	C	T	*	*	*
EP12	*	A	TG	G	-	C	C	*	*	T	*	*	C	---	C	C	*	C	G	*	C	T	*	*	*

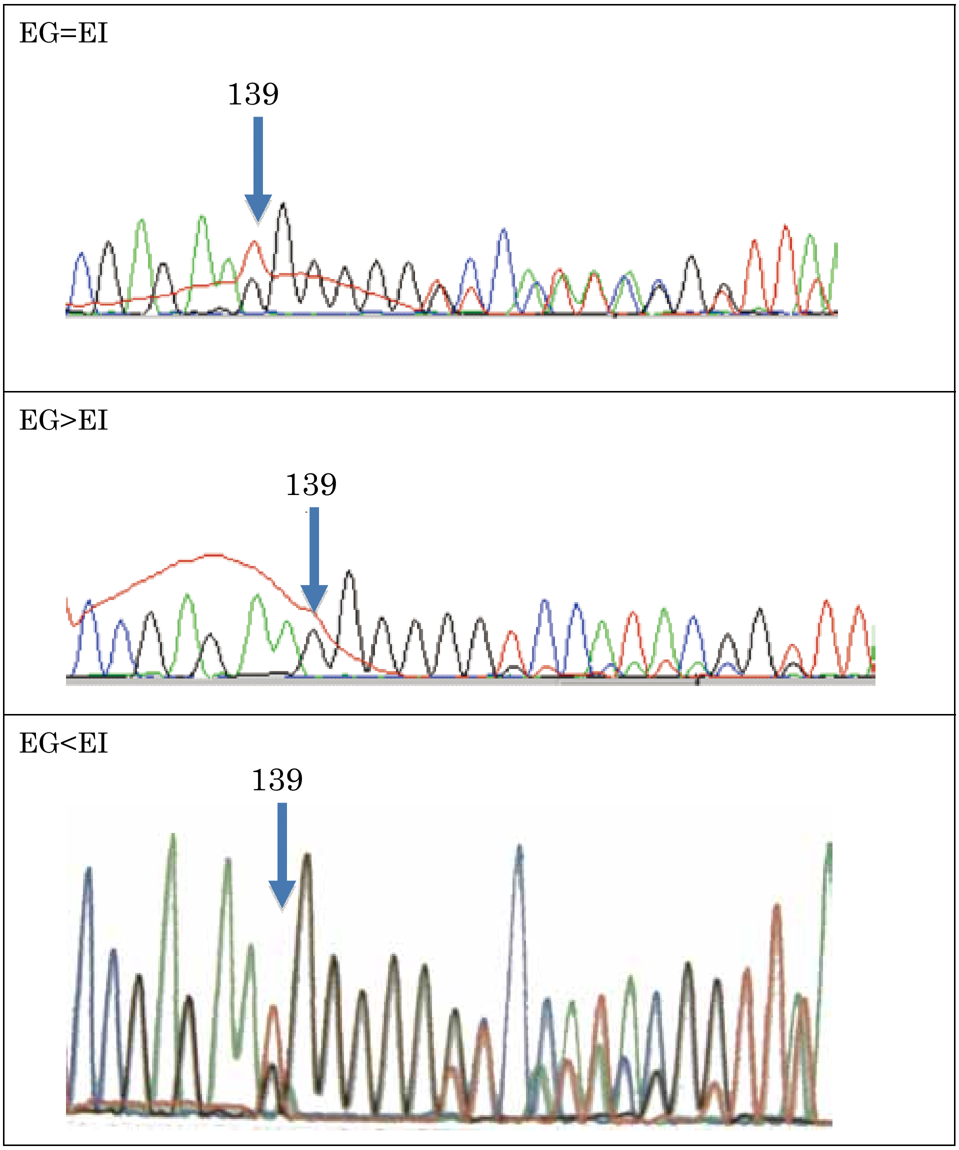
Specimen ID (site)	756	762	773	784	797	800–03	810	844	846	872	875–78	892	912–3	917–19	934	949	951	954–57	985	1026	1030	1105	1131	1132	1134
E. intermedia (China) [a)]	A	C	T	C	T	TTGG	C	G	G	T	TTGG	-	AG	TCG	G	C	T	TTGG	A	A	T	C	G	C	C
E. gerardiana (China) [b)]	*	T	-	T	C	----	A	*	*	C	----	C	CA	---	A	T	C	----	G	C	A	T	A	*	T
E. gerardiana (Pakistan) [c)]	*	T	-	T	C	----	A	*	*	C	----	C	CA	---	A	*	C	----	G	C	A	T	A	*	T
EG1, EG2, EG3, EG4, EG5, EG6, EG10	*	T	-	T	C	----	A	A	*	C	----	C	CA	---	A	*	C	----	G	C	A	T	A	*	T
EG7	*	T	-	T	C	----	A	*	A	C	----	C	CA	---	A	*	C	----	G	C	A	T	A	T	T
EG8	*	T	-	T	C	----	A	*	A	C	----	C	CA	---	A	*	C	----	G	C	A	T	A	T	T
EG9	*	T	-	T	C	----	A	*	A	C	----	C	CA	---	A	*	C	----	G	C	A	T	A	T	T
EG11	*	T	-	T	C	----	A	A	*	C	----	C	CA	---	A	*	C	----	G	C	A	T	A	*	T
EG12	*	T	-	T	C	----	A	R	R	C	----	C	CA	---	A	*	C	----	G	C	A	T	A	Y	T
EP12	*	T	-	T	C	----	A	*	R	C	----	C	CA	---	A	*	C	----	G	C	A	T	A	C	T

*Same nucleotide as in the top column.
-: Deletion, R = A/G, Y = C/T.
Columns in grey are sites where nucleotide variation is found among Nepalese specimens.
[a)] DDBJ/EMBL/GenBank accession number. AY394070 (Long et al. 2004).
[b)] DDBJ/EMBL/GenBank accession number. AY394074 (Long et al. 2004).
[c)] DDBJ/EMBL/GenBank accession number. NJ176183 (Kakiuchi et al. 2007).

a similar sequence to the western specimens. Among the Nepalese *E. gerardiana,* nucleotide variations at 15 positions in ITS1 were found.

Analysis of ITS1 of Nepalese *Ephedra pachyclada* by cloning

Most of the 12 *E. pachyclada* specimens were found and collected along the Kali Gandaki River valley in the Mustang region at relatively low altitudes 2000–3500 m (Table 1, Fig. 1). Except for one specimen, EP12, the specimens showed complex ITS1 signals from the 139th nucleotide by the overlapping of two signals. On the other hand, EP12 had similar sequence to those of Chinese and Pakistani *E. gerardiana* (Table 2). Using several sequence primers (Fig. 2), ITS1 sequences up to around the 500th nucleotide were analyzed. As Figure 3 shows, overlapping signals were deduced to bases in ITS sequence of Chinese *E. gerardiana* and *E. intermedia*, and the intensity of signals from bases in each sequence varied among specimens. ITS1 sequences from the 400th nucleotide of 11 specimens were subjected to cloning to separate these sequences (Table 3). A phylogenetic analysis of ITS1 sequences from the 500th nucleotide to the end of clones from EP2, EP4, EP5 and EP6 together with those of Chinese *E.*



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↓

E. gerardiana : CCGAGAAGGGGGGTCCATACGGTTTA

E. intermedia : CCGAGAATGGGGGGTCCATACGGTTT

Fig. 3. Examples of direct sequencing chromatograms of *Ephedra pachyclada* specimens. Complex ITS1 signals from the 139th nucleotide by the overlapping of signals from two bases deduced either to bases in ITS1 of Chinese *E. intermedia* (EI base) or *E. gerardiana* (EG base) are seen. Top: Chromatogram with equally mixed signals of EI base and EG base. Middle: Chromatogram with EG base dominant. Bottom: Chromatogram with EI base dominant.

Table 3. Cloning analysis of ITS1 sequences of *Ephedra* plants morphologically identified as *E. pachyclada*

Specimen ID	Number of clones analyzed	Number of EG type sequences	Number of EI type sequences	Other
EP1	2	1	1	0
EP2	17	7	8	2
EP3	3	3	0	0
EP4	10	8	1	1
EP5	9	8	1	0
EP6	14	5	9	0
EP7	10	3	7	0
EP8	3	1	2	0
EP9	8	0	8	0
EP10	2	0	2	0
EP11	4	1	3	0

intermedia, *E. gerardiana,* and *E. likiangensis* was performed. Figure 4 shows the result of the analysis by the maximum parsimony method. Seven clones out of 16 obtained from EP2 (Fig. 4a) were monophyletic with Chinese *E. gerardiana* (EG-type) and 8 with Chinese *E. intermedia* (EI-type). A clone, no. 2, positioned in-between EG-type and EI-type. Likewise, most of clones from EP4 (Fig. 4b), EP5 (Fig. 4c) and EP6 (Fig. 4d) were classified into either EG-type or EI-type.

Analysis of ITS2 and *trn L/F* of Nepalese *Ephedra pachyclada*

Table 4 shows sequence alignments of ITS2 and *trn L/F* of individuals morphologically identified as *E. pachyclada*. All of the specimens of ITS2 showed overlapping signals in sequence chromatograms, which were deduced to those of Chinese *E. gerardiana* and *E. intermedia.* Furthermore, two thirds of the specimens analyzed showed complex signals in sequencing chromatograms of *trn L/F* sequences from 459th nucleotide due to overlapping of two types of sequences, with or without deletions at the 459th and the 460th nucleotides. These deletions are found in the sequence of *E. sinica* (Table 4, Long et al., 2004).

Discussion

The ca. 50 *Ephedra* species worldwide are distributed in the semiarid regions of warm-temperate zones (Stevenson 1993, Price 1996). As we reported previously, eight major Chinese *Ephedra* plants could be phylogenetically grouped into three by the comparison of DNA sequence, ITS1 and 2, and *trn L/F,* in which *E. gerardiana* and *E. intermedia* belong to the different respective phylogenetic group (Long et al. 2004). In Nepal, Shrestha recorded two *Ephedra* taxa, *E. gerardiana* Wall. ex Stapf and *E. intermedia* Schrenk & C. A. Mey. var. *tibetica* Stapf (Shrestha 1974), whereas Lewis described four, *E. gerardiana* Wall. ex Stapf var. *geradiana*, *E. gerardiana* Wall. ex Stapf var. *sikkimensis* Stapf, *E. intermedia* var. *tibetica* and *E. pachyclada* Boiss (Lewis 1978). However, based on our detailed anatomical study, two species, *E. gerardiana* and *E. pachyclada*, were reported to be dominant in Nepal (Mikage et al. 1996, 1998). The molecular genetic result in this paper clearly showed that two groups corresponded to the morphological identification. The morphological identification of group of

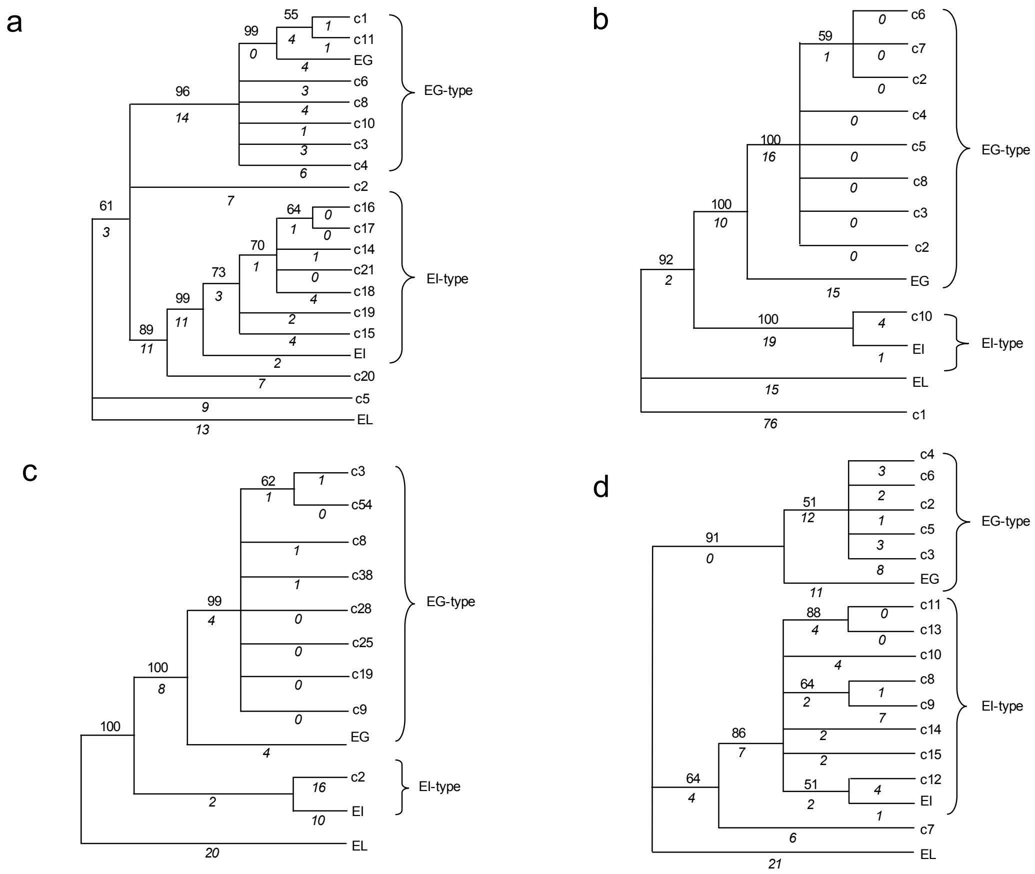

Fig. 4.　Phylogenetic relationship of clones from EP2 (a), EP4 (b), EP5 (c) and EP6 (d). ITS1 sequences from 500 to the end of the clones, numbered with c, were analyzed together with those of Chinese *Ephedra intermedia* (EI), *E. gerardiana* (EG), and *E. likiangensis* (EL). The 50 % majority-rule consensus trees were built on Maximum Parsimony analysis. Numbers above lines are bootstrap values with 100 replicates, and numbers below lines in italics are branch lengths. Tree lengths were 113 (a), 135 (b), 56 (c) and 100 (d).

specimens as *E. gerardiana* was confirmed by having ITS1 sequences similar to those of Chinese and Pakistani *E. gerardiana* (Long et al. 2004, Kakiuchi et al. 2007). Furthermore, there was regional variation in ITS1 sequence among these Nepalese *E. gerardiana.* We found sequence homogeneity in specimens from the western region (S1–4). Although the sequence of the eastern specimens was more various, the extent of sequence variation was not necessarily in accord with the latitude; EG10 from near the eastern border, S10, had a similar sequence to western ones, and two specimens from nearby sites, EG6 and EG11, were not identical. It seems that various geographic factors, such as altitude, and the micro-environment of each habitat, have accelerated genetic diversity. From these data, we did not find two distinctive subspecies of *E. gerardiana*, as Lewis described.

On the other hand, the group of specimens identified as *E. pachyclada*, except specimen EP12, were found to have complex ITS 1 structure consisting of two sequence types, sequences monophyletic with Chinese *E. gerardiana* (EG-type) and Chinese *E. intermedia* (EI-type). Furthermore, judging from relative

Table 4. Alignment of ITS 2 and trn L/F sequences of *Ephedra* plants

ITS 2

	73	84	169	189	205	218	220	230	242	245
E. intermedia (China) [a]	C	A	A	C	G	T	G	G	A	G
E. gerardiana (China) [b]	*	G	G	*	A	C	A	T	G	A
E. pachyclada (Egypt) [c]	T	G	G	T	A	C	A	T	G	A
EG1-12, EP12	*	G	G	*	A	C	A	T	G	A
EP1-8, EP10, EP11	*	R	R	*	*	Y	R	K	R	R
EP9	*	R	R	*	R	Y	R	K	R	R

trn L/F

	9	101	465	466
E. intermedia (China) [d]	-	C	G	T
E. gerardiana (China) [e]	-	A	*	*
E. sinica (China) [f]	-	*	-	-
EG1-12, EP12	T	*	*	*
EP2-4, EP6, EP11	T	*	*	*
EP1, EP5, EP7, EP8, EP9, EP10	T	*	nd	nd

*Same nucleotide as in the top column.

- : Deletion, K=G/T, R=A/G, Y=C/T.

nd: Not determined.

[a] DDBJ/EMBL/GenBank accession number. AY394062 (Long et al. 2004).

[b] DDBJ/EMBL/GenBank accession number. AY394067 (Long et al. 2004).

[c] DDBJ/EMBL/GenBank accession number. AY755779 (Rydin et al. 2004).

[d] DDBJ/EMBL/GenBank accession number. AY423430 (Long et al. 2004).

[e] DDBJ/EMBL/GenBank accession number. AY423436 (Long et al. 2004).

[f] DDBJ/EMBL/GenBank accession number. AY423431 (Long et al. 2004).

intensity of signals in chromatograms (Fig 3) and numbers of emerged clones (Table 3) with respective characteristics, the abundances of these two sequence types varied among specimens. We concluded that Nepalese *E. pachyclada* was not a species with a uniform DNA sequence, but the group of individuals with mixed EI-type and EG-type ITS1 sequences. The Kali Gandaki River Valley in Mustang, where *E. pachyclada* were collected, is a corridor linking the Tibetan Plateau and North Indian Plain. Its environment has been disturbed by climate, animal, and human causes. Curiously enough, we did not find any community of its parental species in the region. It seems that only hybridized offspring may have survived under the harsh climate of the habitat in high altitude such as intense sunlight, low temperatures, strong wind (http://www.dnpwc.gov.np/conservation/conservation-annapurna.asp), and dry condition (http://practicalaction.org/file/region_nepal/ClimateChange1976-2005). In the Northern Region of Pakistan and Sichuan Province in China, which are also located at high altitudes, we found extreme intraspecific variation in ITS 1 of *E. intermedia*, and *E. likiangensis*, respectively but not large hybridized community (Kakiuchi et al. 2007, Inoko et al. 2009). ITS1 sequences of *E. pachyclada* collected from Sinai, Egypt, reported independently by Rydin and Ickert-Bond, were similar to each other, and to *E. geradiana* (Rydin et al. 2004, Ickert-Bond et al. 2004). Thus, Nepalese and Egyptian *E. pachyclada* were different in genetic composition. If they contain ephedrine alkaloids in sufficient amounts (Kondo et al. 1999) and given their presumed ability of adapting to environment at high altitudes, both Nepalese *Ephedra* plants would be hopeful for the medicinal plants resource.

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濱中絵美[a]，大久保圭祐[a]，御影雅幸[a]，垣内信子[b]：**ネパール産マオウ属植物の分子遺伝学的性質**

マオウ科マオウ属植物 *Ephedra* は世界中で 50 ～ 60 分類群が存在すると言われ，各地で薬用にされる有用植物である．多くのマオウ属植物は全草にエフェドリン，プソイドエフェドリンなどのエフェドリン型アルカロイドを含有しており，これらアルカロイドは近代医薬品としても用いられている．日本ではマオウ属植物起原の生薬は葛根湯，小青龍湯といった重要な漢方処方に含まれている．またヒマラヤ地域で実践されている伝統医学であるチベット医学でも用いられており，これらはいずれもマオウ属植物の全草を薬用にしている．本研究では，マオウ属植物の新たな資源を開発する研究の一環として，ネパール産のマオウ属植物を調査し，DNA 解析により分子生物学的な特徴を検討した．ネパールでは野生種として *E. gerardiana* Wall. ex Stapf および *E. pachyclada* Boiss. が自生している．そのうち *E. gerardiana* はヒマラヤ山域の標高 3100 ～ 4500 m の草原に広く生育し，一方の *E. pachyclada* は西部ネパールのムスタン地区のカリガンダキ河沿いの標高 2000 ～ 3500 m に局在している．ITS1 領域の DNA 配列を解析した結果，*E. gerardiana* では，西部の株がすべて同一であったが，東部のより標高が高い地域の株では西部の株と 1 ～ 12 塩基が異なり，かなりの変異が見られた．一方，*E. pachyclada* の ITS1 領域の DNA 配列では，解析した 12 株すべてで 2 種の異なる配列が重

複していることが観察された．このうち，4 株の ITS1 領域をクローニングし，得られたクローンの DNA 配列について系統解析した結果，それらクローンの ITS1 領域の DNA 配列は中国産 *E. gerardiana* あるいは *E. intermedia* Schrenk & C.A.Mey. と同系統の，2 系統に別れることが明らかになった．また各々の系統の DNA 配列を有するクローンの出現比率は株間で異なっていた．ネパール産の両分類群とくに *E. pachyclada* の遺伝的多様性は，ヒマラヤ山中の環境に適応した結果と考えられ，厳しい環境に対応しうる生物資源として有望である．

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